GLUCOCORTICOID ENHANCEMENT OF GENE EXPRESSION

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BACKGROUND OF THE INVENTION

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Cross-Reference to Related Application

This patent application is a divisional of U.S. Serial number 08/709,554, filed September 6, 1996, which claims benefit of priority of U.S. Serial number 60/003,418, filed September 8, 1995, now abandoned.

Field of the Invention

The present invention relates generally to the fields of drug action, cellular regulation and gene therapy. More specifically, the present invention relates to the novel finding that glucocorticoids

enhance reporter gene activity when transfected by cationic lipid or liposomes.

Description of the Related Art

5   Human clinical trials for gene therapy as treatment of diseases caused by genetic defects or by gene dysregulation have shown promise and gained momentum in the last two years. Several diseases which manifest significant symptomology in the lung have been targeted for gene therapy, including cystic fibrosis and lung

10   cancer. These trials have employed either recombinant retroviral or, adenoviral vectors as well as cationic lipids to transport and deliver the gene to the cell. Nonetheless, cellular transfer and subsequent gene expression is low and thus therapeutic levels of gene expression may be lacking. Moreover, immune responses developing against viral

15   vectors may limit their use. While cationic lipids are less efficient at delivery than the adenoviral vectors, newer chemical designs have produced cationic lipids that are greatly improved over the original designs. Several animal and human trials have shown that, at cationic lipid concentrations typical for transfection, no negative side effects

20   or immune response developed.

          Delivery   of   gene therapy to the lung by aerosol

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allows genes to be delivered directly to the target tissue. Several groups have demonstrated aerosol delivery and transfection of animal lungs *in vivo* using reporter gene DNA coupled to cationic liposomes. The salient features noted in these studies were the absence of toxicity

5 and a duration of gene expression of approximately 1 month. Gene expression was still relatively low; even modest transfection in mouse lungs required at least 0.5 to 12 milligrams of highly purified DNA. As reported, gene therapy by these methods would not be feasible for humans.

10 Another approach to increasing transfection efficiency is a greater understanding of plasmid uptake and the factors which influence expression of the transfected gene in the target tissue. In recent investigations as to the role of inflammation on gene transfection in lung cells, it was found that an exposure of A549

15 human lung carcinoma cells to the immune stimulator lipopolysacchride or the cytokine IL-1β before transfection with pCMVβgal-DMRIE/DOPE reduced the level of β-gal protein below that seen in the cells treated with medium only.

The prior art is deficient in the lack of effective means of

20 delivering therapeutic levels of transfected genes. The present

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invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

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Little is known about the mechanism by which cationic lipid-DNA complexes are taken up by the cell or, the fate of the complexes within the cell. Even less is known about factors *in situ* that may influence uptake, or the consistent delivery and expression

10 of DNA once in the tissue, especially in patients with chronic lung inflammatory disease or any other immune process. The present invention demonstrates two findings which have a substantial effect on tranfection in cell culture and which have parallels *in vivo*. First, the present invention demonstrates that the cytokine IL-1β and the immune

15 stimulator lipopolysacchride (LPS) suppress transfection/expression of pCMVβgal transfected into A549 human lung cancer cell lines or primary rat lung cells by cationic lipid. Secondly, anti-inflammatory topical glucocorticoids such as beclomethasone dipropionate (BEC) reverse the inhibitory effects of IL-1β and lipopolysacchride and even

20 enhance expression of reporter genes above and beyond expression

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seen in untreated transfected cells, i.e., not treated with lipopolysacchride or IL1β, etc. The effect is specific to glucocorticoids, as opposed to other types of steroids but not to a particular glucocorticoid. This effect may also be specific to glucocorticoids as anti-inflammatory agents as this effect was not seen when cells were pre-treated with another immunosuppressant cyclosporine A. Glucocorticoid-mediated boost in transgene activity is independent of promoter, reporter gene and cationic lipid used. The mechanism by which glucocorticoids enhanced expression of reporter genes does not involve increased plasmid-lipid uptake, but rather an intracellular mechanism which does not involve new protein synthesis. In addition, pretreatment of primary rat lung cells with synthetic topical glucocorticoids, in the absence of lipopolysacchride or IL-1β, prior to transfection enhanced the level of β-gal protein over untreated controls. The present invention describes studies concerning the mechanism of glucocorticoid-enhanced transfection of plasmid DNA. Thus, the present invention has direct relevance to the use of gene therapy *in vivo*.

In one embodiment of the present invention, there is provided a method of increasing the cellular expression of a gene in a

biological tissue after delivery of said gene in an appropriate vector to an animal, comprising the step of: administering to said animal a pharmacologically effective dose of a glucocorticoid in an amount sufficient to increase the cellular expression of said gene.

5   In another embodiment of the present invention, there is provided a method of treating a pathophysiological state in a human by increasing the cellular expression of a gene in an appropriate vector after delivery of said gene into a biological tissue of a human in need of such treatment, comprising the step of: administering to said

10   human a pharmacologically effective dose of a glucocorticoid in an amount sufficient to increase the cellular expression of said gene.

        Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the

15   purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

20   So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which

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will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows that lipopolysacchride and IL-1β suppress β-galactosidase (β-gal) activity in A549 cells transfected with pCMVβgal-cationic lipid. A549 cells were treated with either medium, 0.5 μg/ml of lipopolysacchride or 100 U/ml recombinant human IL-1-β for 4 hours. After treatment, cells were transfected with 1 μg/ml pCMVβgal- 4 μg of DMRIE/DOPE. β-gal activity was determined using a (CPRG) colorimetric microtiter assay specifically measuring βgalactosidase activity. Results represent the average and standard deviation from 3 experiments. Fold-change = β-gal activity in lipopolysacchride or IL-1 samples/β-gal activity in medium-treated samples.

Figures 2A-2 show that glucocorticoids enhance β-gal activity in transfected A549 cells. A549 cells were treated for 4 hours

with glucocorticoids. **Figure 2A** shows the dose response of the transfected cells to beclomethasone, dose range from 10^{-7} to 10^{-6} M. **Figure 2B** shows that several other topical glucocorticoids also induced enhanced βgal activity at 10^{-6} M: Budesonide, BUD; Flunisolide, FLUN; Beclomethasone-dipropionate-dilauroyl Phosphatidylcholine, beclomethasone-DLPC. After treatment, cells were transfected with 1 µg/ml pCMVβgal- 4 µg of DMRIE/DOPE. β–gal activity was determined using the microtiter assay. Results represent the average and SD from 3 experiments. Fold-change = β–gal activity in glucocorticoid-treated cells/β–gal activity in medium-treated cells.

Figure 3 shows that beclomethasone reverses the inhibitory effect of lipopolysacchride and IL-1β on β–gal activity in transfected A549 cells. A549 cells were treated for 4 hours with medium only, beclomethasone (10^{-6} M), IL-1β (100 U/ml) or lipopolysacchride (0.5 µg/ml). After the first 4 hours, supernatant was removed and cells were treated with either, medium, IL-1β, lipopolysacchride or beclomethasone, as designated an additional 4 hours (i.e., beclomethasone, lipopolysacchride; beclomethasone for first 4 hours, lipopolysacchride for the second 4 hours). After the second treatment, cells were transfected with 1 µg/ml pCMVβgal- 4 µg

of DMRIE/DOPE. βgal activity was determined using the microtiter assay. Results shown represent the average and SD from 2 experiments. Fold-chang = β–gal activity in beclomethasone, lipopolysacchride, IL-1 or combination treated cells/β–gal activity in

5   medium treated cells.

Figure 4 shows that beclomethasone enhances β–gal activity in primary rat lung cells. Primary rat lung cells were isolated from enzymatically digested rat lungs and plated at 1.0 X 10^5 cells/well in cell well tissue culture dishes. Cells were pretreated with

10   beclomethasone, lipopolysacchride (0.5 μg/ml) or beclomethasone+lipopolysacchride (4 hours beclomethasone +4 hours lipopolysacchride) in a concentration range of 10^{-7}- 10^{-6} M for 4 hours then transfected with 3 μg of DNA and 12 μg of DMRIE/DOPE. At 48 hours, β–gal activity was determined by CPRG microtiter assay.

15   Results represent the average and SD from 3 (*2) experiments. Fold-change = β–gal activity in beclomethasone, lipopolysacchride, beclomethasone+lipopolysacchride-treated-cells/βgal activity in medium-treated cells.

Figures 5A-5C show that the beclomethasone-mediated

20   enhancement is not limited cationic lipid, vector promoter or vector

reporter genes and is specific to glucocorticoids. A549 cells were treated for 4 hours with beclomethasone or other steroids (**Figure 5B**, steroids: estrogen, E2; Progesterone, PROG; Cholesterol, CHOL). After treatment, cells were transfected with, in **Figure 5A**, 1 μg/ml

5 pCMVβgal and 3 μg/ml DOSPA/DOPE, in Figure 5B, 1μg/ml pSVβ and 4 μg/ml DMRIE/DOPE or 1 μg/ml pCMVβ-gal and 4 μg/ml DMRIE and **Figure 5C**, 1 μg/ml pCMVHICAT and 4 μg/ml DMRIE/DOPE. βgal activity was determined using the microtiter assay. Chloramphicol acetyl transferase (CAT) activity was determined by TLC chloramphicol

10 acetyl transferase assay where isotope ^{14}C acetylated forms of chloramphenicol were resolved by TLC and ^{14}C-incorporation determined by betascanner set to detect ^{14}C. Results in **Figure 5A** and **Figure 5C** represent the average and SD from 3 experiments. Results in **Figure 5B** are representative of 3 experiments. Fold-change

15 = βgal or CAT activity in beclomethasone or steroid-treated cells/βgal or chloramphicol acetyl transferase activity in medium treated cells.

Figure 6 shows that beclomethasone does not increase plasmid uptake in A549 cells. A549 cells were pretreated with medium or beclomethasone at the dose specified for 4 hours, then

20 transfected with ^{3}H-thymidine-labeled pCMVβgal. Two hours post-

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transfection, cells were washed, lightly trypsinized and pelleted by centrifugation.   Pellets were then lysed, resuspended, and liquid scintillation cocktail added. ^{3}H-thymidine-labeled pCMVβgal uptake (CPM) per sample was determined.   Results represent the average and

5   standard deviation from 4 experiments and each experimental point was performed in duplicate.   Fold-change = CPM beclomethasone-pretreated/CPM medium pretreated.

Figure 7 shows the kinetics of beclomethasone-enhanced reporter gene expression.   A549 cells were treated for various times

10   with 10^{-6} M beclomethasone or medium.   Cells were transfected cells were transfected with 1 μg/ml pCMVβgal- 4 μg of DMRIE/DOPE.   βgal activity was determined using a CPRG microtiter assay.   Results represent the average and SD from 2 experiments.   Fold-change = βgal activity in beclomethasone-treated cells/βgal activity in medium-

15   treated cells.

Figure 8 shows that protein synthesis is not required for beclomethasone-mediated enhancement of β–gal activity.   Cells were treated with either medium or CHX (10 μg/ml) for 30 minutes.   Cells pretreated with medium were subsequently treated for 4 hours with

20   either medium or beclomethasone at 10^{-6} M.   Cells pretreated with

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CHX were subsequently treated with either CHX or 10^{-6} M beclomethasone+CHX for 4 hours. Cells were transfected with 1 µg/ml of pCMVβgal and 4 µg/ml DMRIE/DOPE. β–gal activity was determined using a CPRG microtiter assay. Results represent the average and SD from 2 experiments. Fold change BEC= β–gal activity in beclomethasone-treated cells/β-gal in medium-treated cells. Fold-change beclomethasone+CHX = β–gal activity in beclomethasone+CHX treated cells/β–gal activity in CHX-treated cells.

Figure 9 shows that beclomethasone increased steady-state mRNA levels in A549 cells. A549 cells were treated with 10^{-6} M beclomethasone or medium for 4 hours. Cells were transfected with 1 µg/ml pCMVβgal plus 4 µg/ml DMRIE/DOPE. At times specified (hours) after transfection, total RNA was harvested, converted to cDNA and specific βgal messages amplified by RS-PCR as described below. RS-PCR samples were resolved on a 1% TAE agarose gel and transferred to nylon filter. Filter was hybridized to a ^{32}P-labeled βgal probe. Filter was analyzed by betascanner and bands of the appropriate size counted. Fold-change = βgal CPM in beclomethasone treated cells/βgal CPM in medium-treated cells.

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DETAILED DESCRIPTION OF THE INVENTION

In the present invention, the following abbreviations may be used: lipopolysacchride: *Salmonella typhimurium* lipopolysacchride; IL-1ß: Interleukin-1-ß; GC: glucocorticoids; E2: estradiol; PROG: progesterone; CHOL: cholesterol; BUD: Budesonide; BEC: beclomethasone dipropionate; FLUN: flunisolide; DLPC: dilauroyl phosphatidylchoine; β-gal: *Escherichia coli* beta-galactosidase; CAT: chloramphicol acetyl transferase; DMRIE/DOPE: N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecytoxy)-1- propanaminium bromide dioleoyl phosphatidylethanolamine; DOSPA/DOPE: 2,3 dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N dimethyl-1-propanaminium trifluoroacetate: dioleoyl phosphatidylethanolamine; DMEM: Dulbecco's minimal essential medium; FBS: fetal bovine serum; CHX: cycloheximide; RS-PCR: RNA-specific polymerase chain reaction; CPRG: Chlorophenored-ß-D-galactopyranoside; cAMP: cyclic adenosine monophosphate; CREB: cAMP responsive element binding protein;

The present invention is directed to a method of increasing the cellular expression of a gene in a cell after delivery of said gene to an animal, comprising the step of: administering to said animal a

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pharmacologically effective dose of a glucocorticoid in an amount sufficient to increase the cellular expression of said gene.

The present invention is also directed to a method of treating a pathophysiological state in a human by increasing the cellular expression of a gene after delivery of said gene into a biological tissue of an animal in need of such treatment, comprising the step of: administering to said animal a pharmacologically effective dose of a glucocorticoid in an amount sufficient to increase the cellular expression of said gene.

Generally, any glucocorticoid may be used in the method of the present invention but apparently not any anti-inflammatory since the effect was not seen in cyclosporine A-treated cells. Representative examples of useful glucocorticoids include hydrocortisone, prednisone, prednisolone, triamcinolone, betamethasone, budesonide, flunisolide and dexamethasone. The glucocorticoids may be either synthetic or non-synthetic glucorcorticoids. Generally, the glucocorticoid is administered in a dose of from about 0.6 mg/kg to about 50 mg/kg, dependent upon which glucocorticoid, as a diffence in potency exists and, whether a physiologic or pharmacologic dose is to be delivered. In the methods of the present invention, the glucocorticoid may be a lipid

soluble form, an ethanol soluble form or a water-soluble form, any of which may be incorporated into a liposome.

Generally, the glucocorticoid may be administered in a fashion that optimzes the ability of the glucocorticoid to enhance the activity of the gene delivered. For example, the glucocorticoid may be administered concurrently with the delivery of said gene, prior to delivery of said gene or after delivery of said gene. The route of administration may be any that is desirable in the art including aerosol, intravenous, intraperitoneal, etc.

The methods of the present invention are efficacious regardless of the biological tissue to which the gene is delivered. For example, the activity of a gene may be enhanced if it is delivered to tissues such as the liver, leukocytes, lung, gastrointestinal tract, kidney, skeletal muscle, smooth muscle, neurological tissue, skin cells, cancer cells, eye, bone marrow and tumors.

The delivery of the gene may be by any route. For example, the gene may be delivered by injection, oral administration dermal or aerosol administration. In another embodiment, the glucocorticoid may be encapsulated in a liposome, neutral or charged as is well known in the art. Alternatively, the gene may be dissolved in a solvent such as ethanol.

The methods of the present invention are efficacious in any animal, either human or non-human. That is, although the methods of the present invention have primary utility in humans various veterinary uses will be apparent to those having ordinary skill in this

5   art.

In the methods of the present invention, the gene may be transfected by a known method. Representative examples of methods of transfecting a gene into a biological tissue include viral transfection, cationic lipid transfection, and targeted gene therapy

10   utilizing a receptor and a cationic amine such as poly-L-lysine. In fact, any gene's activity may be enhanced using the instant methodology whether a recombinant gene, a native gene, a cDNA or an oligomer. The glucocorticoid enhances the activity of a vector at the promoter or at some cell regulatory step prior to translation.

15   The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

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EXAMPLE 1

<u>Cells and cell culture</u>

A549 were obtained from ATCC. A549 were maintained in

5   Dulbecco's minimal essential medium (DMEM, GIBCO-Life

Technologies) plus 10% low lipopolysacchride-containing, defined

fetal bovine serum (FBS, Hyclone), 200 mM L-glutamine and 50 µg/ml

of Gentamicin. For most transfections, cells were plated at 3 X 10^4

cells/ml in 12 well cluster dishes (Corning). This concentration of

10   cells provides a monolayer approximately 30% confluent at 24 hours

post cell transfer.

Primary rat lung cells were prepared as follows. Lungs

were harvested from adult Sprague-Dawley female rats and finely

minced. The minced lung was resuspended in a solution of 0.5X

15   trypsin in Hank's balanced salt solution for 1 hour and then a solution

containing 100 U/ml of collagenase (*C. perfringens*, IV, Sigma

Chemical), 100 U/ml DNase I (Sigma), in Dulbecco's minimum

essential medium (DMEM) with 10% fetal bovine serum (FBS). Tissue

was incubated on a rocker at 37°C for 1.5 hours. Dissociated cells

20   were filtered through several layers of sterile gauze and plated in 6

well cluster dishes (Corning) at 1.0 X 10^6 cells/ well in DMEM + 10%

FBS. Cells were incubated in a humidified 37°C 5% CO_2 incubator for

13-24 hours prior to transfection.

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EXAMPLE 2

Chemicals and reagents

Cycloheximide, lipopolysaccharide (lipopolysacchride, *S.*

10 *Typhimurium*). Budesonide (BUD) and flunisolide (FLU) were also

purchased from Sigma Chemical Co, St. Louis, MO. All glucocorticoids

were dissolved in absolute ethanol. Beclomethasone dipropionate was

the gift of Orion Pharmaceuticals, Kuopio, Finland. Beclomethasone, in

a neutral liposome, dilauroyl phosphatidylcholine (DLPC, Avanti Polar

15 Lipids, Birmingham, AL) was made by dissolving 0.5 mg of

beclomethasone and 25 mg of DLPC in tertiary butanol at 37° C.

Samples were then flash frozen in ethanol and dry ice and lyophilized.

Liposomes were reconstituted in sterile, endotoxin-free water. Human

recombinant IL-1β was purchased from Genzyme Corp. (Cambridge,

20 MA).

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EXAMPLE 3

<u>cDNA</u>

pCMVßgal and pCMVHICAT were obtained from Genzyme Corp. and pSVβ was purchased from Clontech (CA). Plasmid DNA was extracted and purified using the Qiagen column purification system (Qiagen, Chatsworth, CA). Most of the lipopolysacchride was removed from the plasmid preparation the use of a E-TOX column (Sterogene, CA). Plasmid was evaluated for endotoxin using the LAL kit from Biowhitaker/Microbiological Associates (Bethesda, MD). DNA concentration was determined by A_{260} reading and comparing similar concentrations of plasmid-purified DNA with $CsCl_2$-purified DNA. One OD unit of absorbance at 260 nm is equal to 50 μg/ml of DNA.

EXAMPLE 4

<u>Lipids</u>

2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N dimethyl-1-propanaminium trifluoroacetate (DOSPA), dioleoyl

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phosphatidylethanolamine (DOPE) (DOSPA/DOPE, Lipofectamine) was purchased from GIBCO/BRL. N-(2-hydroxyethyl)-N,N-dimethyl-2, 3-bis(tetradecytoxy)-1-propanaminium bromide (DMRIE)/DOPE was obtained from Genzyme Corp. (Framingham, MA).

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EXAMPLE 5

DNA-Cationic liposome preparation

10 DNA-lipid ratio was optimized as follows. Plasmid cDNA (2.5 μg) was combined with various concentrations of cationic lipid in water to generate a panel of DNA:lipid ratios. DNA-lipid mixtures were allowed to incubate for 15 minutes at room temperature and the complexes were resolved by electrophoresis on a 1% agarose gel in 1X

15 Tris-acetate (40 mM)-EDTA (2 mM) (TAE) buffer. Optimal ratios were considered the concentration of DNA:lipid where all DNA was bound by lipid and therefore retained at the gel origin. Ratios were confirmed by transfection of A549 cells as described below. Optimal DNA:lipid ratios were confirmed for each lipid and plasmid

20 combination and, between each batch of DNA and lipid. For

DOSPA/DOPE the optimal DNA:lipid ratio by weight was 1 µg DNA: 3 µg

of lipid and for the DMRIE/DOPE, 1 µg DNA: 4 µg lipid.

5   **EXAMPLE 6**

Transfection

All transfections were performed in OPTIMEM (GIBCO-Life

Technologies). One µg of pCMVβgal was combined with 4 µg of

10   DMRIE/DOPE or 3 µg of DOSPA/DOPE in 1 ml of OPTIMEM and

incubated for 15 minutes at room temperature. Cell monolayers were

washed twice in serum-free medium then overlaid with 1 ml of

transfection mixture and incubated for 2.5 hours at $37^{O}C$ in a

humidified, 5% CO_2. DNA-liposome overlay was replaced with DMEM

15   plus 10% FBS and cells were incubated for 48 hours. Cell lysates were

harvested and assayed for total protein according to the

manufacturer's instructions (BCA assay, Pierce Chemical, Rockfield, IL)

and β-galactosidase activity was determined using a Chlorophenored-β-

D-galactopyranoside colorimetric microtiter assay as described (CPRG)

20   assay (Boerhinger-Mannheim, Germany). Where specified, cell

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monolayers were washed with PBS then fixed with 2% formaldehyde-0.2% glutaraldehyde and stained with X-gal (40 µg/ml, 5'-3'Inc. Boulder, CO) in 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide and 2 mM magnesium chloride.

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EXAMPLE 7

DNA-lipid uptake studies

10   ^{3}H-thymidine-labeled pCMVβgal plasmid was prepared by adding 1 mCi of ^{3}H-TdR (^{3}H-TdR, 74GBq/mmol, Amersham) to a 25ml overnight culture of pCMVβgal-transformed DH5α strain of *Escherichia coli*. Labeled plasmid was isolated using Qiagen tip columns (100µg size, Chatsworth, CA) per manufacturer's instructions. Saturable

15 binding of the labeled DNA was determined as follows. Serial dilutions, from 1 µg-0.125 µg of ^{3}-H-TdR-labeled DNA was added to A549 cells and incubated for either 2, 6 or 24 hours. Monolayers were washed, rinsed with 1X trypsin, lightly trypsinized and cells pelleted by centrifugation. Pellets were lysed in 100 µl of lysis buffer

20 (0.1 M TRIS and 0.5% Triton X-100), liquid scintillation cocktail added

(BCS, Amersham) and ^{3}H-plasmid label quantitated by liquid scintillation. For uptake experiments, A549 cells were pretreated with either glucocorticoids or medium for 4 hours. Cells were treated with the saturable concentration of ^{3}-H-labeled plasmid plus lipid for 2.5

5 hours. Uptake was determined as described above.

EXAMPLE 8

10 <u>RNA-specific PCR (RS-PCR)</u>

RS-PCR was performed as described and is well known in the art. Briefly, cells were pre-treated with beclomethasone at 10^{-6} M and transfected as described above. After the incubation was complete, cells were lysed using acidified guanidinium isothiocyante

15 (Fluka Chemical), containing 0.75M sodium citrate and 1% sarkosyl. RNA was harvested using the RNAeasy system (Qiagen, Chatsworth, CA). Total RNA quantity was determined by A_{260} and, 0.5 µg of the RNA was run on a gel to determine RNA integrity. Using 0.3 µg of total RNA, cDNAs were prepared using MMLV reverse transcriptase (GIBCO,

20 Life Technologies), substituting primer T30D20-gal

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(GAACATCGATGACAAGCTTAGGTATCGATACACCTCGCGGAAACCGACAT)

(SEQ ID NO: 1) for oligo dT. This primer contains 20 base pairs complementary to the 3' end of the mRNA and 30 irrelevant bases. In addition, each reaction was spiked with 2.5 µCi of ^{32}-P α-dCTP as a

5   tracer. Reactions were incubated at 37°C for 1 hours. When incubation was complete, DNA-RNA hybrids were harvested using the Qiaquick spin PCR purification kit (Qiagen, Chatsworth, CA) to remove primer and unincorporated isotope. One µl of cDNA was spotted to duplicate nitrocellulose filters and counted by LSC.

10   For PCR, 25,000 CPM were used for each sample. PCR was otherwise performed as described. The PCR cocktail contained 500 mM KCl, 50 mM TRIS-HCl, pH 9.0,50 mM NaCl, 10 mM MgCl$_2$, 200 µM dNTPS and 1 Unit Taq DNA Polymerase (Promega, Madison WI). Primers: 5βgal (GAGAATCCGACGGGTTGTTACT) (SEQ ID NO: 2) and

15   T30-gal (GAACATCGATGAACAAGCTTAGGTATCGATA) (SEQ ID NO: 3), which represents the terminal 30 nucleotides of the T30D20-gal oligomer, were used at a concentration of 1.25 µM. PCR cycling was optimized for this combination of primers and templates (33 cycles). Upon completion of PCR, products were resolved on a 1% TAE agarose

20   gel, then blotted to nylon filter. Filters were hybridized with ^{32}P-

labeled pCMVβgal probe overnight and washed to remove non-hybridized counts. Blots were evaluated on a Betagen betascanner and CPM for bands of the correct size were determined.

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EXAMPLE 9

Effect of bacterial lipopolysacchride and interleukin-1-β on transfection efficiency

10   Lipopolysacchride in the bacterial cell is a potent immunomodulator, capable of triggering a cascade of events including cytokine production and cellular recruitment. In previous studies, A549 cells were shown to respond to both lipopolysacchride and IL-1-β by upregulation of IL-1β mRNA (lipopolysacchride) as well as the

15   induction of IL-8 mRNA (IL-1β).

In the present invention, A549 cells were treated with either 100 U/ml IL-1-β or 0.5 µg/ml lipopolysacchride for 4 hours, then transfected with pCMVβgal-DMRIE/DOPE. As can be seen in Figure 1, both IL-1-β and lipopolysacchride decreased β-gal activity

20   significantly, relative to the medium treated A549 cells.

EXAMPLE 10

Topical glucocorticoids enhanced ß-galactosidase activity in pCMVßgal-cationic lipid-transfected in A549 cells

A549 cells were pre-treated with synthetic, topical glucocorticoid beclomethasone, in a dose range between 10^{-7}-10^{-6} M or medium for 4 hours prior to transfection. Enhancement of 2-4 fold was consistently seen with beclomethasone in the range 5 $X10^{-7}$ M to 10^{-6} M, with only slight enhancement seen at 10^{-7} M (Figure 2A). In addition, other topical steroids such as budesonide, FLUN and a neutral liposomal form of beclomethasone, beclomethasone-DLPC, also enhanced β-gal activity in A549 cells (Figure 2B). Beclomethasone-DLPC, at 10^{-6} M also enhanced β-gal activity to a similar degree as budesonide or beclomethasone in ethanol (Figure 2A), which showed that the liposome moiety did not interfere with this effect. The FLUN consistently demonstrated less of an enhancing effect, which is consistent with the potency of the glucocorticoids.

EXAMPLE 11

Glucocorticoid-treatment reverses the suppression of gene expression
by IL-1-β and lipopolysaccchride

5   Beclomethasone appeared to have reversed the undesirable

effects of these two immunomodulators. To demonstrate this

phenomenon more particularly, A549 cells were pretreated with 10^{-6}

M beclomethasone for 4 hours, followed by a treatment with

lipopolysacchride or IL-1-β for 4 hours prior to transfection with

10   pCMVβgal-DMRIE/DOPE. As seen previously, the IL-1β and

lipopolysacchride suppressed the degree of β-gal expression and the

beclomethasone enhanced β-gal activity by 2.5-fold. In cells, pre-

treated withbeclomethasone, then treated with either IL-1β or

lipopolysacchride (beclomethasone, lipopolysacchride;

15   beclomethasone, IL-1β), β-gal expression exceeded that seen in

untreated cells by 2-fold and approached the level of the

"beclomethasone only" pre-treated cells, suggesting that the

beclomethasone blocked the inhibitory effects of IL-1β or

lipopolysacchride.

20   Since many of the patients targeted for gene therapy may

have chronic gram negative bacterial infections, the inhibitory effects of the lipopolysacchride and IL-1β would most probably be in place before delivery of the gene or the beclomethasone. To determine whether or not the enhancing effect of the beclomethasone on β-gal expression could overcome the suppression of βgal activity in lipopolysacchride or IL-1β pretreated cells, A549 cells were pre-treated for 4 hours with lipopolysacchride or IL-1β, then treated for 4 hours with beclomethasone prior to transfection with pCMVβgal-DMRIE/DOPE (IL-1β, BEC; lipopolysacchride, BEC).

Figure 3 shows that even when cells were treated with lipopolysacchride or IL-1β, such that the inhibitory process is already established (see lipopolysacchride, IL-1β only), beclomethasone dipropionate treatment not only restored βgal activity, but still augmented βgal activity above what was seen in medium treated, transfected cells. The level of β-gal activity in the lipopolysacchride- or IL-β pretreated, then beclomethasone-treated cells did not quite achieve the level of enhanced β-gal activity seen in cells pre-treated with beclomethasone only, suggesting that some aspect of lipopolysacchride or IL-1β-induced inhibitory activity remained. Thus, the present invention demonstrates that, even during existing

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infections, beclomethasone treatment improves gene transfection by DNA-cationic lipid.

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EXAMPLE 12

Characterization of glucocorticoid-mediated enhancement of reporter gene activity

To determine whether or not the enhancement of βgal

10 activity by glucocorticoids was a more generalized phenomenon, primary rat lung cells were isolated and pretreated with either medium or glucocorticoids for 4 hours prior to transfection with pCMVβgal-DMRIE/DOPE. As can be seen in Figure 4, a composite of results from several different preparations of rat cells, a 2-fold enhancement of β-

15 gal activity was seen. Primary rat lung cells appeared to be more sensitive to beclomethasone, than were the A549 cells, with the optimum enhancement in βgal activity found at 10^{-7} M. Similar to what was observed in A549 cells, lipopolysacchride-suppressed βgal activity. Also, beclomethasone pretreatment, followed by

20 lipopolysacchride stimulation, restored βgal activity to that of the

medium pre-treated cells; enhancement above the control was slight. Thus, glucocorticoids also reversed the inhibitory effects of lipopolysacchride on β-gal activity in a heterogeneous population of primary rat lung cells, suggesting that glucocorticoids may also

5   enhance transfection of similar cell types *in vivo.*

The glucocorticoid effect was not specific to cationic lipid, DMRIE/DOPE, as β-gal expression was also enhanced in cells pre-treated with beclomethasone then transfected with pCMVβgal-and DOSPA/DOPE, another cationic lipid (Figure 5A). Moreover, the effect

10   was not specific to the CMV promoter in the vector as the glucocorticoid-mediated enhancement was observed when A549 cells were pre-treated with beclomethasone dipropionate and transfected with pSVβ-DMRIE/DOPE, a vector that contains the SV40 promoter (Figure 5B). Finally, the glucocorticoid-mediated enhancement of gene

15   expression was not limited to the reporter gene β-gal as similar results were seen when the reporter gene was chloramphenicol acetyl transferase (CAT) (Figure 5C).

To rule out the possibility that other steroids might also augment βgal activity, A549 cells were treated with various doses of

20   either estrogen (estradiol), progesterone, cholesterol or

3 0

beclomethasone for 4 hours prior to transfection with either pCMVβgal or pSVβ using cationic lipid DMRIE/DOPE. Enhancement of β-gal was only seen in the beclomethasone treated cells, showing that, in A549, the enhancing effect was glucocorticoid-specific (Figure 5B).

5 The dose response of the beclomethasone-mediated enhancement using an alternate lipid, promoter or reporter was similar to that seen with pCMVβgal plus DMRIE/DOPE in A549 cells, with 5×10^{-7} to 10^{-6} M producing maximal enhanced reporter gene activity.

These combined results of the studies of the present 10 invention demonstrate that this effect related to glucocorticoid action on cells was a general phenomenon and was not specific to a particular glucocorticoid, reporter gene, promoter, lipid, or cell line. The effect was dose-dependent in primary lung cells demonstrating a greater sensitivity to the beclomethasone. Since the effect was not 15 specific to particular glucocorticoids, beclomethasone dissolved in ethanol was used for the remainder of the studies described herein.

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EXAMPLE 13

<u>Mechanism of beclomethasone dipropionate-mediated enhancement of reporter gene expression: vector uptake</u>

5   One possibility for enhanced reporter gene expression was increased uptake of vector. This possibility was addressed in two ways: determining the number of β-gal stained cells vs. activity and, radiolabeled-plasmid-cationic lipid uptake.

For stained cells, duplicate cell cultures of A549 cells were
10   treated for 4 hours with beclomethasone dipropionate or medium only, then transfected with pCMVβgal using DMRIE/DOPE. At 48 hours, one set of cultures was lysed and βgal activity determined while the other set was fixed with neutral buffered formalin and stained with β-gal (duplicate wells for each treatment). Stained cells were counted in
15   a 1X1 cm^2 area. As can be seen in TABLE I, while β-gal activity was increased in the β-gal assay, the number of cells stained for β-gal was similar between the medium-pretreated cells and the beclomethasone dipropionate pre-treated cells, suggesting that the enhanced β-gal expression was not due to a greater number of cells taking up plasmid.

20

TABLE I

TREATMENT	FOLD-CHANGE, X-GAL STAINING	FOLD-CHANGE, βGAL ACTIVITY
DNA-DMRIE/DOPE	1	1
BEC, 10^{-6} M, DNA-DMRIE/DOPE	1.5 ± 0.7	3

Since it was possible that the staining technique was not sensitive enough to allow detection of increased plasmid uptake, this question was approached by comparing uptake of radiolabeled plasmid in beclomethasone-treated or medium-treated cells. To assure that the plasmid was basically unaltered by the labeling, the plasmid was metabolically labeled by adding ^{3}H-thymidine to the bacterial culture during plasmid preparation (*vide supra*). After determining saturable binding and kinetics of uptake for the labeled material, A549 cells were treated for 4 hours with several concentrations of beclomethasone dipropionate, then transfected with ^{3}H-labeled DNA-DMRIE/DOPE. At 2 hours, cells were trypsinized, lysed and uptake of radiolabeled plasmid determined by LSC. The results presented in Figure 6 confirm what was seen by staining;

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beclomethasone does not appear to enhance uptake, and thus, beclomethasone must function to enhance β-gal activity by some other mechanism.

5

EXAMPLE 14

Kinetics of GC-mediated enhancement of transfected β-gal expression

To define the time course by which glucocorticoid-induced

10   enhancement of βgal activity, A549 cells were pre-treated for various

timed periods with either medium or 10^{-6} M beclomethasone to

determine kinetics of this effect. As can be seen in Figure 7, a

minimum of 3-4 hours of beclomethasone exposure was required in

order to see the enhanced β-gal transfection. Enhanced β-gal activity

15   was maximized by 8 hours pre-treatment and remained maximal for

the 24 hours monitored. The 3-4 hours pre-incubation requirement

plus, the plasmid uptake experiments suggest that an intracellular

synthetic step may be involved in mediating the beclomethasone

enhanced β-gal response rather than a cell surface event.

20

EXAMPLE 15

Intracellular action of glucocorticoid-mediated enhancement of vector activity

5   To demonstrate whether or not beclomethasone-mediated enhancement of β-gal activity required protein synthesis, the protein synthesis inhibitor cycloheximide (CHX) was employed.   Designated A549 cell cultures were pre-treated with medium or CHX to impose a block in protein synthesis.   After 30 minutes, medium-treated cell

10   cultures were treated with either medium or beclomethasone. Those already CHX-treated, were additionally treated with either med+CHX or beclomethasone+CHX for 4 hours.   All cells were transfected with pCMVβgal-DMRIE/DOPE and β-gal activity was determined 48 hours later.

15   As can be seen in Figure 8, CHX moderately suppressed the β-gal activity in control cells, most likely by blocking synthesis of the β-gal protein (comparing the pCMVβ-gal plus and minus CHX).   In contrast, beclomethasone-mediated enhancement of β-gal activity was still seen in cells treated with beclomethasone+CHX.   In fact, the

20   beclomethasone-mediated enhancement of β-gal activity was even

greater in the presence of CHX. These results demonstrate that for beclomethasone to augment β-gal activity protein synthesis was not required.

5

EXAMPLE 16

BEC effect on pCMVβ-gal mRNA levels in A549 cells

To establish kinetics of β-gal gene expression, A549 cells

10   were transfected with pCMVβ-gal-DMRIE/DOPE. At various times after transfection was complete (2.5 hours), total RNA was harvested at T=2, 3, 7, 11, and 24 hours. mRNA was detectable by 2 hours and remained high for the entire period of time examined (data not shown). Once the kinetics of β-gal gene expression were established,

15   A549 cells were pre-treated with medium or 10^{-6} M beclomethasone for 4 hours, then transfected for 2.5 hours with pCMVβ-gal. Total RNA was harvested at T= 0, 2, 3 or 24 hours and thereafter, mRNA levels determined by RS-PCR. While the mRNA levels were high in untreated as well as beclomethasone-treated cells by T=2 hours (38- and 18-fold

20   induction, respectively), by T=3 and T=24 hours a definite increase in

steady-state m RNA levels was detectable in the beclomethasone dipropionate-treated cells (Figure 9). These results demonstrate that either transcription was increased or that the mRNA was stabilized in the cytoplasm by glucocorticoids.

5   The present invention demonstrates that glucocorticoids enhanced expression of reporter genes transfected into cells by cationic lipid-mediated transfection. The glucocorticoid effect was not dependent on a particular cationic lipid, vector promoter, vector reporter gene or cell type. Instead, the glucocorticoids effect was a

10   general phenomenon related to glucocorticoids, as other steroids such as estrogen, progesterone or cholesterol did not enhance reporter gene expression with either of two promoters driving the β-gal gene. Glucocorticoids did not appear to function by increasing plasmid uptake by the cells, but in some fashion, enhanced expression of the

15   reporter gene. A minimum of 3-4 hours of exposure with glucocorticoids was required to see the enhancement of gene expression. New protein synthesis was not required for beclomethasone-augmented reporter gene activity and CHX did not increase gene expression in the absence of beclomethasone

20   dipropionate, suggesting that the superinduction seen in the presence

of CHX may be glucocorticoids-specific.

The glucocorticoid effect was seen in A549, a human lung cancer cell line as well as primary rat lung cell isolates. The glucocorticoid-directed increase in βgal activity$_\circ$ was not seen in COS-1 monkey kidney cells transfected with pCMVβgal-DMRIE/DOPE. It is possible that COS-1 cells lack a receptor for glucocorticoids or lack some other glucocorticoid-sensitive intracellular factor involved in the enhanced response. A cell line which does not respond to the glucocorticoid effect may be useful in clarifying the exact nature of this response.

Most likely, a greater enhancement of β-gal activity by beclomethasone in the presence of lipopolysacchride (Figure 4) would have been seen if the 10^{-7} M beclomethasone concentration was used instead of the 10^{-6} M concentration, which was less effective than was 10^{-7} M in the primary rat cultures. This modification is being examined, along with an intermediate concentration 5 X 10^{-7} M.

Lipopolysacchride was present in the plasmid preparations used and in virtually every other plasmid preparation made in most laboratories. Removal of lipopolysacchride from plasmid is an obstacle that will need to be overcome when using gene therapy

clinically.

Whether the lipopolysacchride is in the plasmid or found pre-existing in inflamed lungs, the ability of the beclomethasone to reverse the inhibitory effects of lipopolysacchride or IL-1β, and possibly other cytokines or immune processes should be of great importance for gene therapy in the clinic.

The mechanism of the glucocorticoid-mediated enhancement of reporter gene expression has only just begun to be elucidated. Glucocorticoids have been shown to have numerous regulatory properties that are dependent on glucocorticoids concentration, cell type, and target gene or intracellular cite. Glucocorticoids may be imposing regulation on the immune response; genes known to be down-regulated by glucocorticoids include IL-1β gene, TNF-α, intracellular adhesion molecule-1 (ICAM-1) as well as structural genes which make up the extracellular matrix such as collagen and stromelysin. Glucocorticoids have been shown to regulate gene by several different mechanisms such as decreasing gene transcription as seen for IL-1β gene but glucocorticoids also affect IL-1β expression at a post-transcriptional step.

Glucocorticoids also upregulate gene expression. The

glucocorticoid receptor after interaction with glucocorticoids, directly binds to regulatory elements, i.e., a specific DNA sequence known as the glucocorticoid response element (GRE). In a glucocorticoid-responsive gene promoter the binding of the GC-glucocorticoid

5   receptor complex upregulates transcription of the gene. Alternatively, the glucocorticoid receptor has been shown to bind to other transcription factors, such as c-*fos* or c-*jun* which, in the absence of glucocorticoid-receptor, activate gene transcription. By the glucocorticoid-receptor-c-*fos* or c-*jun* association, c-*fos*, c-*jun* are

10   unavailable to activate responsive genes.

In the present invention, neither of the promoters used have the more common GREs, as determined by searching the gene bank of sequences. Messenger RNA half-life might have been extended due to the glucocorticoid-mediated inhibition of an mRNA-specific

15   endonuclease but since CHX did not superinduce gene expression in the absence of beclomethasone, the inhibition of the endonuclease must also have some specificity for glucocorticoids. Alternatively, a cofactor which enhances transcription or translation, a co-factor for which new protein synthesis is unnecessary, may be positively

20   modulated by glucocorticoids, thus enhancing production of the reporter gene protein.

A recent study by Liu *et al.* showed dexamethasone (DEX) augmented reporter gene activity, when it was coupled to a segment of the somatostatin promoter. The mechanism was shown not to involve the classic GRE in the promoter but instead, glucocorticoids receptor

5   showed cooperative activity with a protein, cAMP regulatory element binding protein, (CREB) known to bind another transcriptional element, cAMP regulatory element (CRE). Both cAMP and protein kinase A were involved in Dex-mediated enhancement of reporter gene activity. A similar situation has been shown for the PEPCK gene;

10   glucocorticoids upregulated and cAMP augmented transcription. In the present invention, the glucocorticoids effect shown here was not specific to a single promoter region. That is, glucocorticoids enhanced β-gal activity from vectors that had either the SV40 promoter or the CMV promoter. It is unlikely that both of these

15   promoters have similar CRE sequences in their promoters, but this possibility is not known.

Another study showed the positive effects of cell proliferation on transfected gene expression. Similar to what was seen herein, an increase in reporter gene uptake was not observed but a 10-

20   fold increase in luciferase activity was seen in cells stimulated to

proliferate by cell injury. It is unlikely that glucocorticoids may have induced cell proliferation in the studies of the present application, since the cell density of the A549 or lung cells was such that the cells were in log growth phase for the entire incubation period of 48 hours.

5   Glucocorticoids have been used in the clinic for many years and are considered safe and effective. Patients with chronic lung inflammatory disease are good candidates for topical treatment with glucocorticoids delivered as an aerosol. In addition, glucocorticoids treatment has been shown to improve overall lung function in patients

10   with cystic fibrosis, especially if the treatment is given early in the course of disease.

Considering the safety of glucocorticoids and the cost of gene therapy, which consists of expensive preparations of DNA and cationic lipid and, considering the potential side effects of the

15   repeated delivery of large quantities of DNA-lipid makes the present invention all the more intriguing, both in terms of gene therapy as well as the molecular biology of glucocorticoids.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain

20   the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures,

treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art

5   which are encompassed within the spirit of the invention as defined by the scope of the claims.